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CHOLERA TOXIN FEEDING DID NOT INDUCE ORAL TOLERANCE IN MICE AND ABROGATED ORAL TOLERANCE TO AN UNRELATED PROTEIN ANTIGEN[1]

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The feeding of protein antigens to mice results in a state of tolerance when feeding is followed by parenteral immunization. Cholera toxin (CT) is a protein that has been used extensively as a potent oral immunogen for mucosal IgA responses, but CT feeding also stimulates a substantial plasma IgG antibody response. This latter finding prompted us to study whether or not CT induces oral tolerance. Mice were fed 5 mg keyhole limpet hemocyanin (KLH) or 10 μg CT at least twice before parenteral immunization with 1 μg KLH or CT in alum i.p. Plasma and intestinal secretions were collected at intervals. The specific IgG or IgA antibody in the samples was measured by ELISA. Although KLH feeding did induce oral tolerance, CT feeding did not induce oral tolerance in any of three mouse strains tested or at any dose of CT given orally. The feeding of the B subunit of CT did not result in oral tolerance either. When both CT and KLH were fed together, CT was able to abrogate oral tolerance to KLH, an antigenically unrelated protein. Moreover, feeding CT along with KLH stimulated secretory IgA anti-KLH responses, whereas no such IgA responses were found when KLH was given alone. Thus, in these experiments with protein antigens, IgA immunization and oral tolerance were reciprocally linked and did not occur simultaneously. CT appears to abrogate oral tolerance and to stimulate secretory IgA responses by altering the regulatory environment in gut-associated lymphoid tissue, shifting it toward responsiveness.

Systemic tolerance has been found to occur after the feeding of multiple different types of antigens to a variety of animal species. Specific systemic unresponsiveness in mice after protein feeding has been demonstrated for IgG and IgE antibody responses, antigen-specific T cell proliferation, and delayed skin reactions (1–3). The degree and duration of unresponsiveness to proteins depends on the dose fed, but a state of partial unresponsiveness can persist for months after a single large bolus feeding. There is a small but measurable amount of serum antibody present after the antigen feeding; however, there appears to be no direct correlation between the amount of this antibody and the degree of systemic unresponsiveness (4). Feeding protein antigens to previously immunized mice blunts the response to subsequent parenteral boosting (5) and under certain experimental conditions has abolished the existing serum antibody response altogether (6).

Cholera toxin (CT)[3] is a protein antigen that has been used extensively in the study of IgA immunization (7–9). It appears to be the most potent oral immunogen known. We recently found that CT feeding also stimulates a substantial plasma IgG response (10), although we and other investigators (4) have not found this to be the case after the feeding of other protein antigens. Although CT is unusual in this respect, we thought it possible that CT might be simply a stronger antigen than other proteins but qualitatively the same, i.e., the response to CT might be more pronounced but in the same direction as the response to other proteins. Thus, although the feeding of CT induced an IgG antibody response in plasma, subsequent immunization with CT parenterally might result in the same type of significantly reduced systemic IgG anti-CT response, i.e., oral tolerance, as is found after the feeding of other protein antigens (1, 2, 4).

These experiments were designed to ask the question: Does CT feeding result in oral tolerance? The experiments show that CT did not induce oral tolerance. Moreover, administering CT together with the unrelated protein antigen keyhole limpet hemocyanin (KLH) induced an IgG anti-KLH response in plasma, stimulated an IgA anti-KLH response in intestinal secretions, and abrogated oral tolerance to KLH, a pattern of response identical to that found to CT after CT was fed alone.

MATERIALS AND METHODS

Animals. C3H/He mice were obtained from Charles River, Cambridge, MA. BALB/c and C57BL/6 mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Mice were obtained at age 4 to 6 wk and were used at age 6 to 8 wk.

Antigens. CT and the B subunit of CT were obtained from Sigma Chemicals, St. Louis, MO. KLH was obtained from Calbiochem-Behring, San Diego, CA.

Feedings and immunization. Intragastric feedings were administered by using a blunt-tipped feeding needle (G. Tiemann and Sons, Long Island City, NY). The desired amount of antigen was administered in 0.5 ml of 0.2 M $NaHCO_3$. Mice were parenterally immunized by injecting 1 μg of the relevant antigen in 20% Maalox (v/v) i.p. with an identical booster dose given subsequently.

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[3] Abbreviations used in this paper: CT, cholera toxin; ELISA, enzyme-linked immunoassay; KLH, keyhole limpet hemocyanin; GALT, gut-associated lymphoid tissue.

Collection of samples. Blood was obtained from the retro-orbital plexus of ether-anesthetized mice by using heparinized capillary tubes. After transfer to small plastic centrifuge tubes and centrifugation for 5 min at 11,000 × G in a microcentrifuge, the plasma was aspirated off and stored at −20°C until assay.

Intestinal secretions were collected by using a lavage technique that has been described (11). Briefly, the mice were placed on a 12 x 12-cm^2 square of galvanized wire mesh, which was in turn placed on a 100 x 15-mm plastic petri dish containing 3 ml of a protease inhibitor solution. The mice were kept on the wire mesh by inverting over them a 600-ml glass beaker. Four doses of 0.5 ml of the lavage solution were given intragastrically at 15-min intervals by using a blunt-tipped feeding needle. The lavage solution consisted of NaCl 25 mM, Na_2SO_4 40 mM, KCl 10 mM, $NaHCO_3$ 20 mM, and polyethylene glycol (average m.w. = 3350) 48.5 mM. Thirty minutes after the last dose of the lavage solution the mice were given 0.1 mg pilocarpine i.p. A discharge of intestinal contents occurred regularly over the next 10 to 20 min. The protease inhibitor solution was of a mixture of soybean trypsin inhibitor, EDTA, and phenylmethylsulfonyl fluoride (11). The samples were stored frozen at −20°C until assay.

Measurement of antibody. Antibody in plasma and secretions was measured by enzyme-linked immunoassay (ELISA) as described (11). Briefly, antigen was coated on the wells of a 96-well polystyrene Immulon plate (Dynatech, Rockville, MD). After an incubation with sample or standard mouse antibody, the wells were then incubated with rabbit anti-mouse IgG or rabbit anti-mouse IgA, followed by an incubation with sheep anti-rabbit globulin coupled to alkaline phosphatase. Finally, 0.1 ml of *p*-nitrophenylphosphate (1 mg/ml; Sigma Chemical Co., St. Louis, MO) in 10% diethanolamine buffer at pH 9.8 was added to each well, and the color development after a 30-min incubation at room temperature was measured at OD 405 in a MR 580 micro-ELISA autoreader (Dynatech Instruments, Santa Monica, CA). A standard curve was constructed for each assay, and the values of the samples were interpolated by using a program based on Rodbard's four parameter logistic model (12) on an Apple II Plus Microcomputer. As a standard for anti-CT antibody, a reference hyperimmune serum was used, and the antibody concentration expressed in units of standard activity: one unit of anti-CT antibody is defined as that amount that gave an absorbance at OD 405 equivalent to a 1×10^6 dilution of the reference CT antiserum. Similarly, one unit of anti-B subunit antibody is defined as that amount that gave an absorbance at OD 405 equivalent to a 1×10^6 dilution of the reference B subunit antiserum. The reference standard for anti-KLH antibody was an affinity-purified antibody obtained from the serum of hyperimmunized mice; the results of anti-KLH antibody are thus expressed as micrograms per milliliter.

The rabbit anti-mouse IgA and IgG used in the assay were obtained by immunizing rabbits with mouse IgA or IgG myelomas (Litton Bionetics, Rockville, MD). Each of these antisera was solid-phase absorbed against the other mouse isotypes. Specificity was verified by showing that these antisera did not bind to other isotypes in the ELISA. Because the reference antisera are predominantly IgG antibodies, when measuring specific IgA antibody in intestinal secretions, rabbit anti-mouse IgG was used for the standards but rabbit anti-mouse-IgA was used for the unknowns. To ensure that the readings of these two rabbit antisera were comparable, we titered each antiserum in preliminary experiments against an equivalent amount of either IgG or IgA coated to plastic. The titer of each rabbit antiserum that gave an OD 405 reading of 1.0 when the wells were coated with 10 ng of the relevant immunoglobulin isotype was determined; these titers were then used in all assays. The slopes of dilution curves obtained with these two antisera were parallel. The sheep anti-rabbit globulin was affinity purified on rabbit γ-globulin-Sepharose, then coupled to alkaline phosphatase (Type VII; Sigma) with the use of glutaraldehyde. In preliminary experiments this antibody conjugate was shown not to bind to mouse immunoglobulin.

Measurement of total IgA present in intestinal secretions was by sandwich ELISA. The wells of the Immulon plate were coated with affinity-purified goat anti-mouse IgA (Cappel Laboratories, Pittsburgh, PA). After the incubation with samples and standards, the wells were sequentially incubated with a rabbit anti-mouse IgA and sheep anti-rabbit globulin coupled to alkaline phosphatase. A quantitated reference mouse serum (Meloy Laboratories, Springfield, VA) was used as a standard. All the other aspects of the assay were the same as those described above for the measurement of specific antibody.

Statistics. Antibody levels were converted to logarithmic values for calculation of geometric means and standard errors. The significance of differences between experimental groups was tested by using the Mann-Whitney two-sample rank test (13).

RESULTS

Oral tolerance to KLH but not to CT. Groups of C3H/He mice were fed twice with KLH 5 mg or CT 10 μg 21 and 7 days before i.p. priming with KLH or CT. A booster dose of KLH or CT was given i.p. 14 days later. Samples of plasma were collected 1 wk after the booster dose and assayed for IgG anti-KLH or IgG anti-CT by ELISA. As shown in Figure 1, the feeding of KLH before i.p. immunization resulted in an 85% decrease in the response to KLH immunization ($p < 0.001$). In contrast, feeding of CT did not reduce the response to i.p. immunization with CT. In fact, in this experiment, CT feeding primed for an anamnestic response to the parenteral immunization.

Lack of oral tolerance to CT in multiple mouse strains. Mice of three different strains—C3H/He, BALB/c, and C57BL/6—were fed with CT 10 μg 14 days and 7 days before parenteral immunization. Control groups of each strain were fed saline at the same times. All the mice were primed with CT 1 μg in alum i.p. and were given a similar booster dose 28 days later. Figure 2 shows

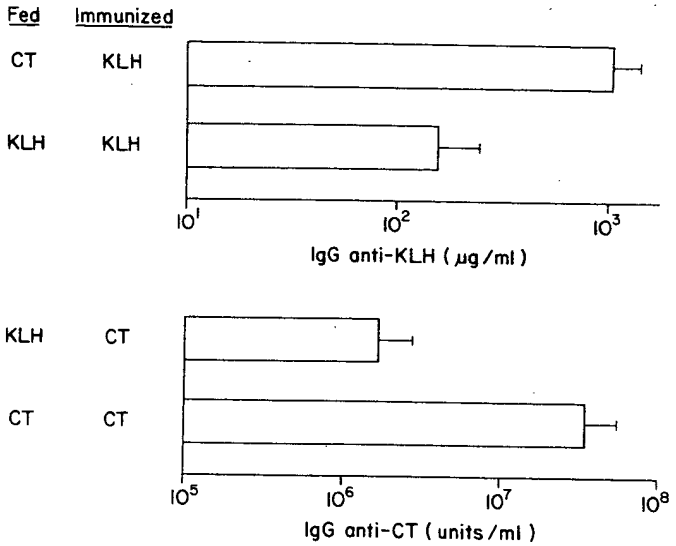

Figure 1. Oral tolerance to KLH but not to CT. Mice were fed with KLH 5 mg or CT 10 μg, 3 and 1 wk before priming i.p. with 1 μg of KLH or CT in alum. Booster dose was given 14 days later. Bars represent geometric mean ×÷ SE antibody response of each group of mice 1 wk after booster dose. *Top*, plasma IgG anti-KLH; *bottom*, plasma IgG anti-CT. Each group contained five mice.

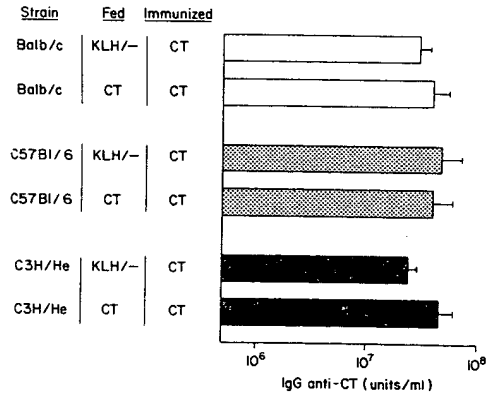

Figure 2. Lack of oral tolerance to CT in multiple mouse strains. Mice were fed on days −14 and −7, primed on day 0 and boosted on day 28. Each bar represents geometric mean ×÷ SE of plasma IgG anti-CT level in group 7 days after booster dose. Each group contained five to six mice. Control groups receiving either KLH or saline and then immunized with CT have been combined (denoted KLH/−).

the plasma IgG anti-CT level 1 wk after the booster dose. None of the strains developed oral tolerance after CT feeding.

The lack of oral tolerance to CT is not dose dependent. Although feeding an optimal dose of CT to mice clearly did not induce oral tolerance, it seemed possible that feeding a suboptimal dose of CT might have a different effect. Therefore, groups of C3H/He mice were fed 10 μg, 1 μg, 0.1 μg, or no CT, 14 days and 7 days before parenteral immunization. Again, these mice were primed with CT i.p. and were given a booster dose 28 days later. Figure 3 shows the IgG anti-CT response in these groups at intervals before and after the priming and booster doses. The mice fed CT did not have a significantly lower plasma IgG anti-CT level than that of mice fed saline at any dose of CT used for feeding.

B subunit of CT does not induce oral tolerance. To determine whether the lack of oral tolerance to CT was due to activation of adenyl cyclase by the A subunit of CT, we fed purified B subunit of CT, which binds to cell surfaces but does not activate adenyl cyclase, to groups of mice which were subsequently immunized with either B subunit or CT. The plasma IgG anti-B subunit response in these groups was then compared with that in a group of mice immunized with B subunit without prior feeding. The secondary plasma IgG anti-B subunit levels in these groups 1 wk after the booster dose are shown in Figure 4. There was no difference in the IgG anti-B subunit levels of the groups that were fed B subunit as compared with those which were not fed B subunit, i.e., feeding B subunit did not result in oral tolerance. SDS-polyacrylamide gel electrophoresis of the B subunit preparation used in these experiments did not reveal any detectable contamination with A subunit (data not shown).

Abrogation of oral tolerance to KLH by CT. Groups of mice were fed saline, KLH 5 mg, or KLH 5 mg plus CT 10 μg 23, 22, 8, and 7 days before parenteral immunization as shown in Figure 5. Plasma samples were obtained after the feeding but before the immunization. The plasma IgG anti-KLH levels in the mice fed KLH plus CT (9.4×÷ 2.85 μg/ml) was significantly higher than that in the mice fed KLH alone (1.3 ×÷ 2.85 μg/ml, $p < 0.05$) or saline (0.5 ×÷ 1.11 μg/ml, $p < 0.001$).

The mice were primed with CT 1 μg in alum i.p. on day 0 and were given a similar booster dose 28 days later. Figure 5 shows the mean plasma IgG anti-KLH level in each group of mice 1 wk after the booster dose. The first group, fed saline and immunized with KLH, was the positive control. The second group, fed KLH and immunized with KLH, had reduced responses consistent with oral tolerance. The third group, fed KLH plus CT, had responses identical to the first group, which was not fed KLH, i.e., oral tolerance was abrogated. A fourth group, fed KLH plus CT but immunized with CT rather than KLH, had low plasma IgG anti-KLH levels, indicating that KLH plus CT feeding by itself did not stimulate high plasma IgG anti-KLH levels.

Measurement of IgG anti-CT levels in the same groups showed that the feeding of KLH plus CT had no effect on the plasma IgG anti-CT levels either before or after parenteral immunization (data not shown).

Absence of intestinal IgA antibody in mice with oral tolerance. Intestinal secretions were collected at intervals from the same groups of mice shown in Figure 5, and the IgA anti-KLH and IgA anti-CT levels were measured by ELISA. The results are expressed as the amount of specific antibody per microgram of total IgA in the sample in order to correct for variability in recovery of IgA. The results are shown in Figure 6*A*. The groups fed

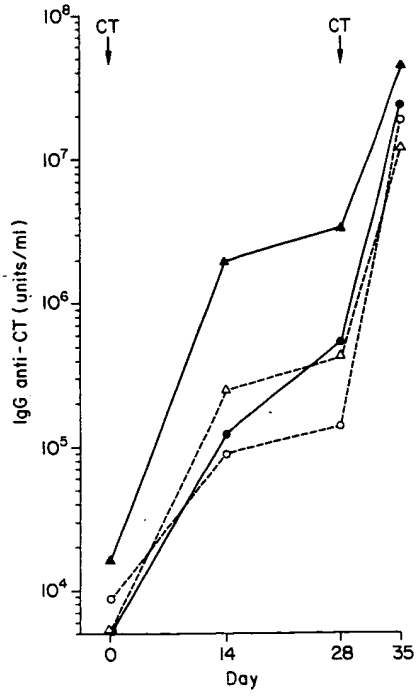

Figure 3. Lack of oral tolerance to CT is not dose dependent. Groups of mice were fed different doses of CT on days −14 and −7, primed on day 0 (*arrow*), and given booster dose on day 28 (*arrow*). Samples were collected at intervals before and after priming and booster doses. Each group contained five mice: —●—, no CT fed; —▲—, CT 10 μg; — —△— —, CT 1 μg; — —○— —, CT 0.1 μg.

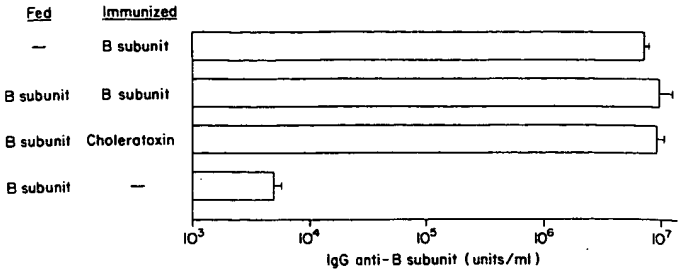

Figure 4. Lack of oral tolerance to B subunit. Mice were fed with B subunit 10 μg on days −23, −22, −8, and −7. They were then primed on day 0 and given booster dose on day 28 with either B subunit or CT, as shown. Bars represent geometic mean ×÷ SE plasma IgG anti-B subunit levels of each group 1 wk after booster dose.

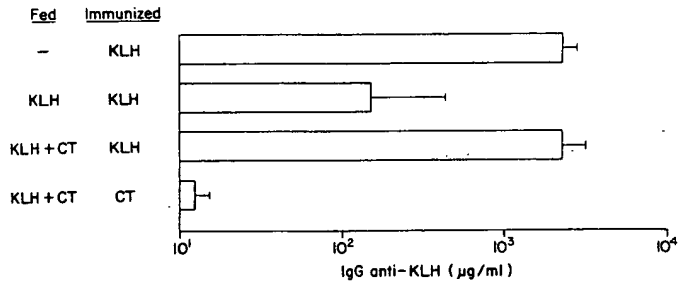

Figure 5. Abrogation of oral tolerance to KLH by CT. Groups of five mice each were fed on days −23, −22, −8, and −7 with KLH 5 mg, KLH 5 mg plus CT 10 μg, or saline. They were primed i.p. on day 0 and given booster dose on day 28 with either KLH or CT, as shown. Bars represent geometric mean ×÷ SE of plasma IgG anti-KLH level of each group 1 wk after booster dose.

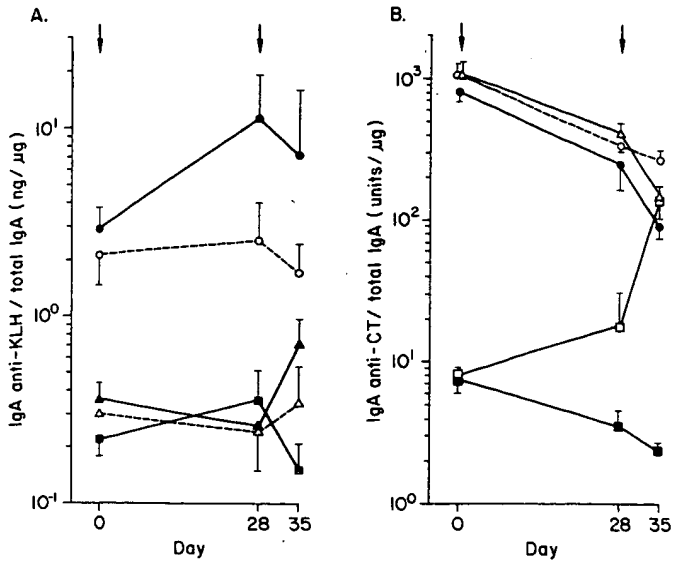

Figure 6. Specific IgA antibody in intestinal secretions after protein feeding. Each group of five mice was fed on days −23, −22, −8, and −7 with KLH 5 mg, CT 10 μg, or both. They were primed with 1 μg of relevant antigen in alum i.p. on day 0 (*arrow*) and were given a similar booster dose on day 28 (*arrow*). Intestinal secretions were obtained at intervals from following groups: —●— fed KLH plus CT, immunized with KLH; — —○— — fed KLH plus CT, immunized with CT; —■— fed KLH, immunized with KLH; — —△— — fed CT, immunized with CT; —▲— fed saline, immunized with KLH; —□— fed saline, immunized with CT.

KLH plus CT had significantly ($p < 0.001$) elevated levels of IgA anti-KLH in intestinal secretions on day 0, collected just before parenteral immunization. In contrast, the group fed only KLH had a level of IgA anti-KLH that was not different from that of control groups not fed KLH. The IgA anti-KLH level in secretions rose further after parenteral priming with KLH in the group fed KLH plus CT but not in the group fed KLH alone.

The intestinal secretions of mice fed either CT alone or KLH plus CT had high IgA anti-CT levels, which tended to decline over time despite the priming and booster doses of CT given i.p. (Figure 6*B*). Thus, in these experiments, each group with a measurable IgA response to the fed protein did not have oral tolerance, and each group without a measurable IgA response to fed protein did have oral tolerance.

DISCUSSION

The experiments detailed above show that the feeding of CT to mice does not result in oral tolerance. At the same time, feeding the protein antigen KLH to mice regularly results in oral tolerance. The lack of oral tolerance to CT was found in multiple different mouse strains and at multiple different doses of CT used for feeding. The lack of oral tolerance after CT feeding does not appear to require the A subunit or adenyl cyclase activating part of the CT molecule, because the feeding of the lectin-like B subunit did not result in oral tolerance either. CT was able to abrogate oral tolerance to the unrelated protein antigen KLH when both were fed to mice together. Moreover, feeding of KLH plus CT stimulated an IgA anti-KLH response in intestinal secretions that did not occur when KLH was fed alone. In these experiments mice had either secretory immunity or oral tolerance, but in no instance were these present simultaneously.

A number of different mechanisms have been found to be operating in oral tolerance, which vary depending on the type of antigen used for feeding. After protein feeding, suppression of antibody responses can be transferred with cells obtained from the Peyer's patch or spleen of fed animals (1, 2) but cannot be transferred by serum. The cell responsible is a radiation-sensitive T cell with a Ly-$1^-2^+3^+$ surface phenotype. These suppressor T cells are present in Peyer's patches as early as 3 days after feeding ovalbumin and are first demonstrable in spleen 7 days after feeding, suggesting that they are first stimulated in gut-associated lymphoid tissue (GALT) and subsequently migrate to other lymphoid tissues (14). Consistent with this idea, splenectomized mice can be tolerized by ovalbumin feeding (4). Although unresponsiveness after protein feeding occurs regularly in most mouse strains tested, NZB/W and C3H/HeJ strains do not develop oral tolerance; in both instances, this appears to be due to defects in the generation of suppressor cells (15, 16). This is the first report of a failure to develop oral tolerance to a protein antigen in mice that do show oral tolerance to other protein antigens.

One of the puzzling aspects about the occurrence of unresponsiveness after protein feeding has been how to reconcile this with the many observations that feeding antigen stimulates mucosal IgA responses. This apparent paradox appeared to be resolved by the studies of Challacombe and Tomasi (17), who showed small but measurable increases in IgA antibody in the saliva of mice that had been fed a single dose of ovalbumin or *Streptococcus mutans* 14 days previously. They were able to show suppression of the systemic immune response in other mice fed similarly. In related studies, other investigators have demonstrated antigen-specific T cell help for IgA responses and T cell suppression for IgG responses shortly after a single feeding of ovalbumin (14), diptheria toxoid (18), or sheep red blood cells (SRBC) (19). This has given rise to the concept that feeding results in simultaneous IgA responses and oral tolerance. These studies have been important conceptually in regard to the isotype-specific regulation of IgA, which has now been more formally demonstrated in T cell cloning experiments (20, 21). However, although mice may have small IgA responses after feeding of protein antigens (just as they have small amounts of serum antibody after protein feeding) associated with the presence of T cell help for IgA and T cell suppression for IgG, our results would indicate that this effect is transient and is not the predominant one, at least not after multiple feedings. This is born out by recent work of Challacombe (22) with a more specific assay of IgA antibody, who has found that single or even repeated weekly doses of ovalbumin or *S. mutans* does not result in significant salivary IgA antibody. Our data, based on a direct and reliable assay of IgA antibody, indicate that feeding of protein antigens other than CT does not stimulate significant IgA antibody in the intestinal secretions of mice, and that oral tolerance and IgA immunization do not co-exist to any significant extent after repeated feedings.

If suppressor cells generated in GALT after protein feeding are responsible for oral tolerance, and the lack of an IgA response coincides with the occurrence of oral tolerance, it seems reasonable to suggest that suppressor cells in GALT are at the same time preventing IgA responses. This would explain the well known lack of

immunogenicity for IgA responses of proteins given orally. Support for this idea comes from the work of Michalek *et al.* (23), who have been examining the effect of bacterial lipopolysaccharide (LPS) on the immune response to antigens given via the intestine. They have found that feeding SRBC to LPS-unresponsive mice stimulates a higher salivary IgA response and in addition primes the mice for a higher anti-SRBC response to subsequent parenteral immunization. In contrast, feeding SRBC to LPS-responsive mice results in a lower salivary IgA response and induces unresponsiveness to a subsequent parenteral injection with SRBC (16, 24). Correspondingly, feeding SRBC to germfree mice primes them for high responses to parenteral immunization with SRBC unless the germfree mice are given LPS intragastrically before SRBC feeding, in which case they develop tolerance to the SRBC (23). These effects have been shown in *in vitro* studies to be due to a nonisotype-specific Ly-1$^-$2$^+$ suppressor T cell. These workers postulate that LPS stimulates suppressor T cell precursors in GALT, which are stimulated by antigen to become the antigen-specific suppressor T cells mediating oral tolerance. Our data obtained after feeding protein antigens has many parallels with this work; whether LPS-stimulated suppressor T cells are involved in the unresponsiveness to KLH and responsiveness to CT found in our experiments is as yet unknown.

The lack of oral tolerance to CT and its ability to abrogate oral tolerance to a second antigen indicates that CT is able to alter the regulatory environment in GALT. More specifically, CT seems able to inactivate or overcome the suppression in GALT that is induced by protein feeding and that mediates oral tolerance. The simultaneous stimulation of IgA responses by CT, both to itself and to KLH (Fig. 6), suggests that at the same time CT inactivates or overcomes suppressor activity in GALT that prevents IgA responses to proteins such as KLH. Earlier studies with CT have shown that it is able to both stimulate and inhibit lymphoid cells (25, 26). The same is true of most substances with adjuvant activity, which can either stimulate or suppress immune responses depending on the proper manipulation of timing, dose, and antigen (27). The effects that CT has on GALT may depend on the status of regulatory activity at the time of exposure, i.e., it may inactivate the major regulatory activity, whether that be help or suppression, allowing the alternate effect to predominate. Thus, it would not be surprising that under the proper circumstances CT might suppress rather than stimulate mucosal and systemic immunity. This would explain a number of observations in which prior exposure to CT, either parenterally or orally, has resulted in the induction of suppression to CT (28).

What is it about the CT molecule that gives it such remarkable qualities? The ability of CT to activate adenyl cyclase and increase cAMP levels in a wide variety of cells is well known. One might expect that this activity would explain the remarkable effects of CT as an enteric immunogen. However, the lack of oral tolerance to the lectin-like B subunit of CT indicates that the A subunit is not the component of the molecule responsible for the lack of oral tolerance to CT. Conversely, the feeding of any antigen that binds to the intestinal mucosa does not necessarily result in a lack of oral tolerance to that antigen. In recent studies, we have found that feeding the lectin concanavalin A (Con A) to mice does result in oral tolerance to Con A (unpublished observations). We infer that the lack of oral tolerance to CT and to B subunit may be due to the binding to or cross-linking of Gm_1 ganglioside molecules on the cell surface membrane of lymphoid cells in GALT (29), thus altering their functional properties, but the question remains open. It is clear that CT is a new and valuable probe for the understanding of oral tolerance and of the relationship between oral tolerance and secretory immunity at the cellular, and ultimately, molecular levels.

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